Genetic characterizations and epigenetic interference to better understand and fight occult Hepatitis B virus infection

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CHAPTER 6

Potential Usage of Hepatitis B Virus X Protein as an Epigenetic Editing Tool for DNA Methylation Alteration and Gene Expression Induction

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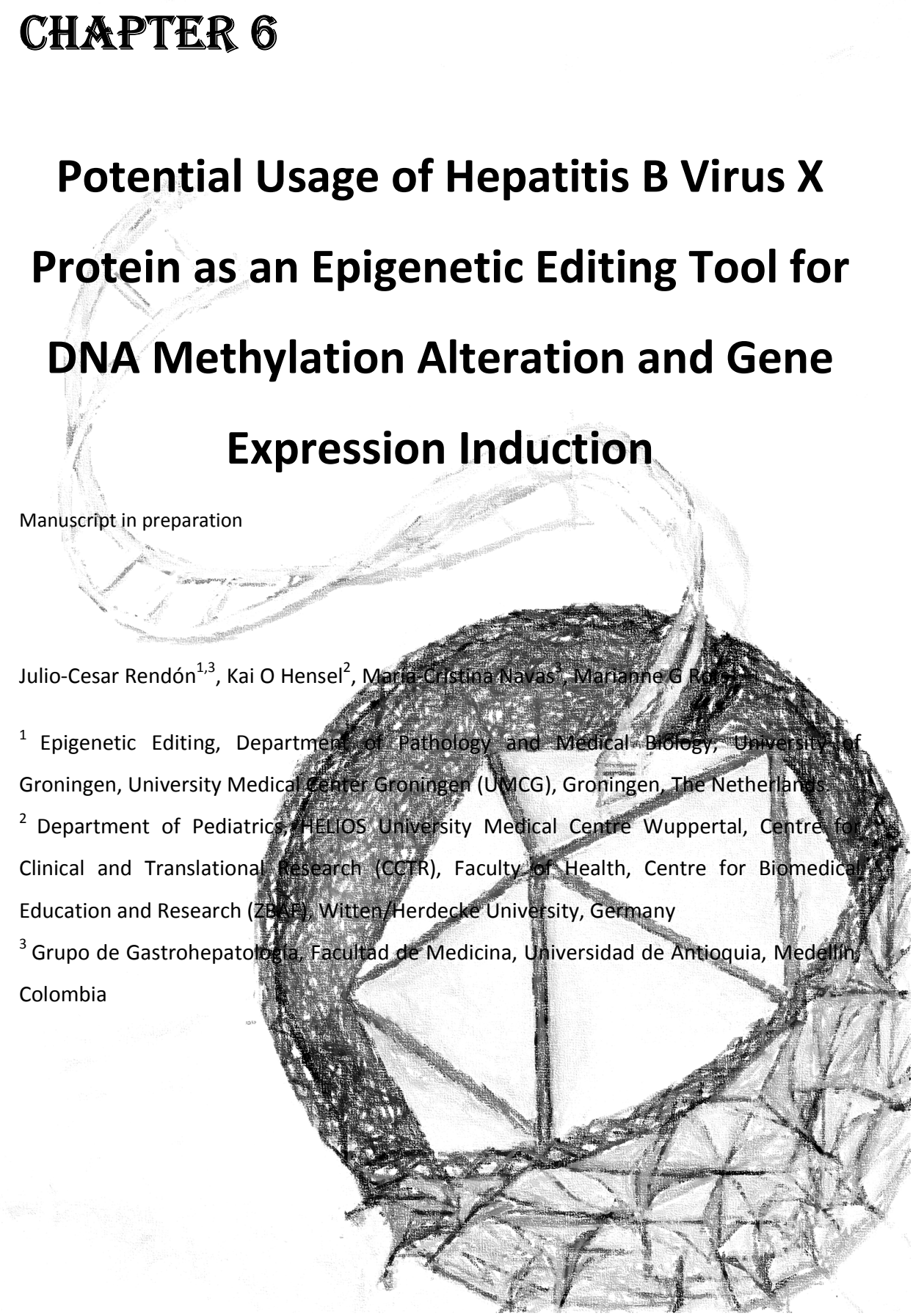

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CHAPTER 6

Abstract

Hepatitis B virus (HBV) is associated with chronic and acute infections and is also related to increased risk of development of severe liver diseases as cirrhosis and hepatocellular carcinoma. Among other proteins, HBV encodes a small 155 amino acid protein, HBx, which is required for viral replication. Importantly, by altering cellular pathways, HBx can induce an environment favorable for maintaining the viral infection. HBx is thought to alter DNA methylation profiles in hepatocellular carcinoma cases, which has been related to altered expression, activity and recruitment of DNMTs and/or other epigenetic enzymes. Here, we addressed the putative role of HBx as an epigenetic modifier by engineering a tetracycline inducible HBx expressing cell line to assess the effect of HBx on whole genome methylation alteration. In addition, we targeted HBx to genomic loci of interest (by fusion to Zinc Finger Proteins or dCas) and evaluated its effect on inducing site specific DNA demethylation or gene expression activation. The results indicate that HBx can induce gene expression from a non-methylated repressed promoter. However as no genome-wide methylation alteration or induced targeted DNA demethylation was observed, further research is required regarding the mechanism of action of HBx.

CHAPTER 6

Introduction

Despite the availability of treatments and vaccine against Hepatitis B virus (HBV) infection, it remains a major health problem with more than 240 million people being chronically infected worldwide. Chronic HBV infection (CHB) even makes the tenth leading cause of death worldwide as more than 780.000 people die every year due to HBV-associated complications, mainly cirrhosis and hepatocellular carcinoma (HCC) (1).

HCC is the fifth most common cancer in men, the ninth in women, and the second most common lethal cancer (2). HBV infection has largely been identified as the most frequent risk factor for HCC development (3) contributing to liver carcinogenesis through multiple mechanisms. Among those mechanisms, genetic instability as a result of viral integration into the host genome has been reported. In addition, expression of viral proteins, be it wild type or mutated/truncated, are known to induce e.g., mitochondrial and endoplasmic reticulum stress, metabolisms changes and altered apoptosis and cell cycle progression(4-7).

The HBV X protein (HBx) is the smallest protein expressed by HBV (155 amino acids) and remains as an enigmatic protein, without homology to any other known gene out of the *Hepadnaviridae* family. Despite its small size, HBx has been shown to feature important roles as a transactivator protein, necessary to achieve sustained viral replication (8-10).

HBx impairs cellular functions by interacting with multiple cellular proteins, which lead to signal transduction alterations, modulation of cellular gene transcription and altered DNA repair, cell proliferation and apoptosis (10-14). Unfortunately, despite reports of several HBx-driven oncogenic mechanisms, it remains challenging to identify the specific biological properties of this protein and its contribution to viral pathogenesis and host carcinogenesis (15, 16).

Several studies have related HBx with the modulation of host and viral epigenetic states, e.g., histone posttranslational modifications (PTMs) and DNA methylation in both viral and cellular genomes (17-19). However, the molecular mechanisms of HBx-mediated epigenetic modifications still need to be elucidated. The lack of both DNA binding domains and enzymatic activity of any kind suggest that HBx does not act as an active epigenetic editor itself, but rather as a scaffold protein, that can recruit or reject epigenetic editors into certain target positions (17, 20).

In this regard, HBx shows a dual effect being able to induce regional hypermethylation on the one hand and global genome hypomethylation on the other hand. This particular effect could be achieved by modulating DNMT expression (21) through its interaction with regulatory proteins such as p53 and the induction of Ras-Raf-mitogen-activated protein kinase signaling cascade involved in DMNT expression (22-25). Additionally, HBx has been shown to modulate DNA methylation in specific promoters by interacting and promoting

or preventing DNA interaction of *de novo* DNA methyltransferase 3A (DNMT3A), which can be related to both up- and downregulation of the affected genes (26).

Moreover, HBx not only modifies the cell microenvironment favoring HBV replication, it also has been shown to specifically enhance viral transcription by favoring histone acetylation of the viral minichromosome, the covalently closed circular DNA (cccDNA). This could be achieved through the recruitment of activating factors such as CBP/p300, and PCAF, while it counteracts cellular repressive marks such as DNA methylation and repressive histone marks (17, 20, 27, 28).

If indeed HBx functions as an epigenetic modulator, HBx can be fused to DNA targeting platforms, and be used to modulate the epigenetic landscape of any desired genomic location in what is known as epigenetic editing (EGE). The EGE concept has been already proved by several studies, which demonstrated the feasibility of writing/erasing epigenetic marks, driving activation or repression of the targeted genes (29-34).

Specific genes as *PLOD2*, *RASSF1a* and *ICAM1* have previously been validated as model genes. *PLOD2* promoter is recognized as an hypomethylated inactive gene in C33A cells, while *ICAM1* and *RASSF1a* promoters are hypermethylated inactive genes in A2780 and MCF7 cell lines (31, 34).

Taking this into account, we evaluate here different possible mechanisms of the transactivation activity of HBx by assessing global and site-specific demethylation, but also methylation-independent, transcription activation.

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Materials and Methods

Plasmid construction

Utilizing the vector pJet_TH24_1,5 (**Chapter 3**), containing a single copy of the HBV genome, genotype F1a; a fragment of 528 bp was amplified using the primers HBx-pMX-Fw and HBx.2-Rv (S1 Table). Because the pJet_TH24_1.5 plasmid features an HBx sequence that is truncated in the last 12 nt, the HBx.2-Rv plasmid was designed to complete the HBx sequence and include a FLAG tag recognition site, a stop codon and restriction sites for *PacI* and *XhoI*.

To obtain both pMX-CD54-HBx and pMX-ZF2-HBx, which contain the coding sequence for zinc finger proteins targeting *ICAM* (CD54) or *RASSF1a* (ZF2) promoters (Table S2), the previously amplified HBx fragment as well as pMX-CD54-TET2 [34] and pMX-ZF2-TET2 [31] were restricted using *MluI* and *PacI* (Thermo Scientific, USA) restriction enzymes.

The pdCas9-HBx plasmid was obtained by restriction of pdCas9-M.SssI-C141S (**Chapter 4**), and pMX-CD54-HBx with the *AscI* and *PacI* restriction enzymes to swop the M.SssI-C141S fragment with HBx. Finally, the pRetroX-Tight-pur plasmid (Clontech, USA) was subcloned to contain the HBx sequence from pMX-CD54-HBx restricted with *MluI* and *EcoRI*.

Cell cultures

Human ovarian carcinoma A2780 cells, breast cancer MCF7 cells, hepatoma HepG2 cells, cervix carcinoma C33A cells and embryonic kidney HEK293T cells were cultured at 37°C and 5% CO_2 in high glucose DMEM medium supplemented with 10% FCS, 2 mM L-glutamine, and 50 mg/ml gentamicin sulfate.

Transfection

C33A cells were seeded in 12 well plates at 300.000 cells/well and transfected using 1 µg of plasmid containing 0.5 µg of pdCas9-VP64 (kindly donated by Keith Joung (Addgene plasmid # 47754)) or pdCas9-HBx and 0.5 µg of sgRNA expressing plasmid(s) (single sgRNA or a combination of several sgRNAs) using Lipofectamine LTX (Invitrogen, USA) in a 2:1:1 (Lipofectamine:DNA:Plus reagent) ratio following the directions from the manufacturer.

Transduction

To produce infectious retroviral particles, the packaging cells HEK293T were co-transfected with both the packaging plasmid pMD2.G and the accessory plasmid pMDLg/pRRE, along with the retroviral vector pMX-IRES-GFP encoding for a zinc-finger (targeting *ICAM1* or *RASSF1a* promoters) and an effector domain (HBx or TET2 or VP64) or the pRetroX-HBx-pur plasmid with a calcium-phosphate protocol [35]. Controls included

pMX-IRES-GFP (empty), and a zinc-finger expressing vector without an effector domain (NoED).

Cells (A2780, MCF7) were transduced using a double transduction protocol [32] with some modifications. Briefly, the virus containing supernatant of (the above mentioned) transfected HEK293T cells was harvested after 48 h and 72h. For each collection, the medium was centrifuged at 1000xg for 10 min, passed through a 0.45 µm FCS filter and supplemented with FCS (final concentration 10%) and polybrene (final concentration 6 µg/ml). Half of the virus containing solution was used to transduce host cells and changed every 8 h to complete total of four medium changes. Assessment of transduction efficiency (GFP) and selection of GFP expressing cells were made 48 h after the last treatment.

Sorting of transduced cells

A2780 and MCF7 transduced cultures were evaluated for GFP expression to select transduced cells by fluorescence activated cell sorting (FACS) as described in **chapter 5.** For each culture, selected cells were grown in appropriate multiple well plates according to the total amount of collected cells. Cultures were maintained increasing the size of the culture container until enough cell were available for both storage freezing and DNA extraction.

Inducible HBx expressing stable cells

HepG2 cells were used to create a double stable inducible system to induce HBx expression with Doxycycline (Dox). The Retro-X™ Tet-On ® Advanced Inducible Expression System (Clontech, USA) was utilized. Direct transduction of both the pRetrox-HBx-pur and the pRetroX-Tet-On Advanced Vector was performed as described above. After transduction, cells were selected with G418 1 mg/mL and puromycin 1 µg/mL while HBx expression was induced using 0.5 µg/ml of Doxycycline (Dox).

Nucleic acid extraction and quantitative real-time PCR

Total DNA was extracted using a noncommercial technique. Up to 6 million cells per pellet were heated at 55°C for 10 min, resuspended in 500 µL of 1x SE buffer (75 mM NaCl + 25 mM EDTA pH 8.0), supplemented with 0.5 µg RNase (Roche, Germany), 0.25 U proteinase K (Roche, Germany), 1% SDS and incubated at 50°C overnight. Next, 222 µL NaCl 6 M and 777 µL of chloroform were added to the cell lysate, rotated for 20 min at room temperature (RT) and centrifuged at 250xg for 15 min.

The upper layer was collected and 1 volume of ice cold Isopropanol was added. Afterwards, the solution was centrifuged at >8000xg for 15 min at RT and the supernatant was discarded. The pellet was washed with 500 µL ethanol 70%, and centrifuged >8000 for

5 min. Finally, the supernatant was discarded and the pellet was dried and resuspended in 30 µL TE buffer.

Total RNA from cell cultures was extracted using GeneJet RNA purification kit (Thermo Fisher, USA) according to the directions from the manufacturer. 100 ng of extracted total RNA was used for reverse transcription utilizing M-MLV (Thermo Fisher, USA) and random hexamer primers (Fermentas, USA).

Quantitative real-time PCR (q-RT-PCR) for PLOD2, GAPDH and HBx mRNA quantification was performed with 10 ng of cDNA using ABsolute QPCR Mix, SYBR Green, ROX (Thermo Fisher, USA) and *PLOD2* (PLOD2-Fw and PLOD2-Rv), GAPDH (GAPDH-Fw and GAPDH-Rv) and HBx (HBx-qPCR-Fw and HBx-qPCR-Rv) primers (S1 Table) [36]. The amplification, detection, and data analysis were performed using the ViiA7 Real-Time PCR system (Bio-Rad, USA).

Methylation analysis by bisulfite sequencing and pyrosequencing

For DNA methylation analyses, 1 µg of total DNA was bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research. USA) following the directions from the manufacturer. Bisulfite-converted DNA was amplified for CpG sites present in promoter regions of *ICAM1* and *RASSF1a* genes using Pyromark PCR kit (QIAGEN, Germany) and specific primers (S3 Table).

ICAM1 promoter was amplified using specific primers for bisulfite treated DNA at final concentration of 0.2 µM being the reverse one biotinylated, the PCR reaction was performed at 94°C 15 min, 40 cycles of 94°C 30 s, 56°C 30 s and 72°C 20 s, and one cycle of 72°C 1 min.

RASSF1α amplification was made using a modified reverse primer which contains an extended tail recognized by a universal biotinylated primer (S3 Table). The PCR reaction was made in two main steps, the first step was conducted utilizing a final concentration of 0.2 µM and 0.04 µM of forward and reverse primer respectively and cycled 94°C 15 min, and 20 cycles of 94°C 30 s, 52°C 30 s and 72°C 20 s. Immediately the reaction was supplemented with the biotinylated universal reverse primer at 0.16 µM of final concentration and continued 20 cycles at 94°C 30 s, 60°C 30 s and 72°C 20 s followed by one cycle at 72°C 1 min.

For all PCR products, 5 µL were separated on 1% agarose gels to evaluate the amplification product. The remaining PCR reaction was used for pyrosequencing according to the directions from the manufacturer with a specific sequencing in the Pyromark Q24 MD pyrosequencer (QIAGEN, Germany). Analysis of the percentage of methylation at each CpG was determined using the Pyromark Q24 Software (QIAGEN, Germany).

Genome-wide methylation levels

Luminometric methylation assay (LUMA) assay was performed as previously described [37,38]. Briefly, 1 µg of total gDNA quantified by the NanoDrop ND 1000 Spectrophotometer (NanoDrop technologies Inc, USA) was run in a 1% agarose gel along with a 1 kb DNA ladder (Fermentas). The sample DNA concentration was estimated by colorimetric intensity. Based on this estimation 500 ng of gDNA was subjected to double restriction using EcoR1+HpaII or EcoR1+MspI.

Sticky ends were filled using PyroMark™ Gold Q24 reagents (QIAGEN, Germany). Nucleotide incorporation was detected by the Pyromark Q24 MD pyrosequencer (QIAGEN, Germany). The percentage of methylation was determined by calculating the ratio of HpaII/MspI for each sample as (HpaII/EcoRI)/(MspI/EcoRI).

Statistical analysis

Mean and s.d. were calculated for each data set. Two- tailed Student's t tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. P values of < 0.05 were regarded as statistically significant.

Results

HBx does not induce global demethylation of host genome

To address a global DNA demethylation effect exerted by HBx, we created a Dox inducible model to control HBx expression in HepG2 cells. The global host genomic DNA methylation status was evaluated using LUMA methodology after two and seven days of sustained induction of HBx. As shown in Figure 1, untreated HepG2 cells showed a 60% of genome-wide methylation, and the treatment with 5-aza cytidine reduced this methylation to 25% ($p<0.05$) and 0.4% ($p<0.01$) on days 2 and 7 respectively, while in the same time points, HBx expression (confirmed by RT-PCR) failed to induce any detectable genomic demethylation.

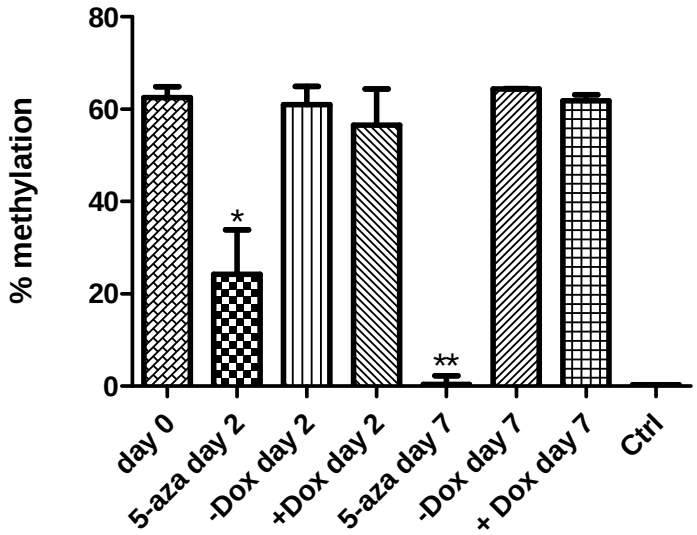

Figure 1. HBx fails to induce global genome alterations. Inducible HepG2 cells expressing HBx were cultured with (+Dox) or without (-Dox) doxycycline and untransduced cells were cultured with 5-azacytidine (5-aza) by 2 and 7 days. Cells day 0, represent inducible cells without induction, and Ctrl refers to control DNA subjected to LUMA. Two-tailed unpaired t-test, *P<0.05, **P<0.01. n=3 independent experiments; mean±SEM.

Targeting HBx to *ICAM1* or *RASSF1a* fails to induce DNA demethylation

To investigate the ability of HBx to induce directed DNA demethylation, A2780 and MCF7 cells were transduced to express previously validated zinc-finger proteins (31, 33) targeting *ICAM1* (CD54) and *RASSF1a* (ZF2) promoters (Table S3) alone (pMX-CD54-NED, pMX-ZF2-NED) or fused with the active domain TET2 (pMX-CD54-TET2, pMX-ZF2-TET2), with VP64 (pMX-CD54-VP62, pMX-ZF2-VP64) or HBx (pMX-CD54-HBx, pMX-ZF2-HBx).

Based on studies published by Chen et al. (34), we initially sorted A2780 GFP expressing cells, collecting between 20% and 95% of the cells. For control untreated cells, methylation percentages of 75-80%, 75-90%, 35-70%, 70-100%, 58-67% and 100% for CpG sites 10-15 of the *ICAM1* region were observed, respectively. No significant changes in DNA methylation levels of the evaluated CpG sites were obtained after transduction of A2780 cells with pMX-Empty, pMX-CD54-NED, pMX-CD54-TET2, pMX-CD54-VP62, and pMX-CD54-HBx (**Fig 2A**).

Similarly, the same set of effector domains was fused to zinc-finger proteins targeting *RASSF1a* promoter region, which is hypermethylated in MCF7 cells. As shown in figure 2B, the methylation level of the *RASSF1a* CpG sites 1-19 in untreated cells range between 29% (CpG 8) and 98% (CpG 6). Upon transduction of cells with pMX-ZF2-NED, pMX-ZF2-TET2, pMX-ZF2-VP64, and pMX-ZF2-

HBx no significant changes in methylation status of the evaluated CpG sites of this promoter were detected.

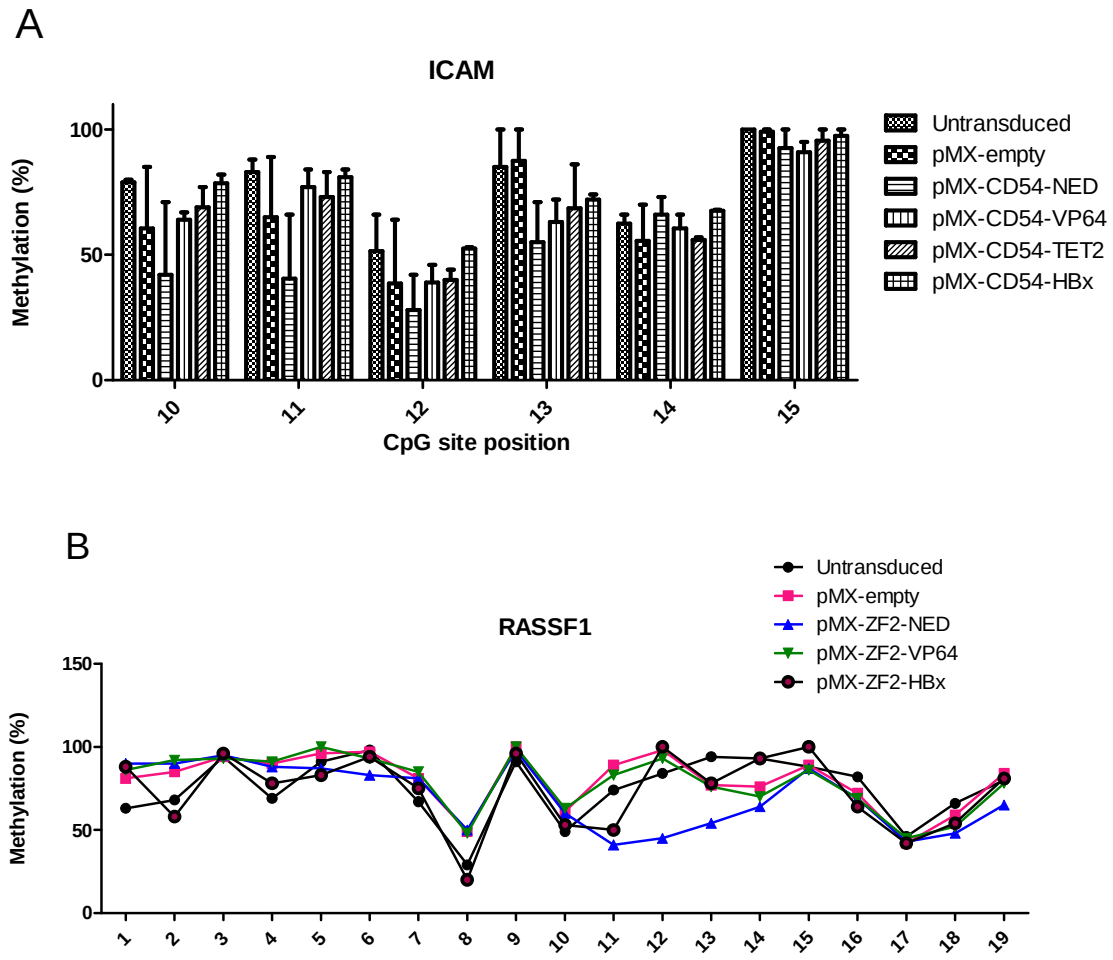

Figure 2. Targeted-HBx fail reduce CpG sites methylation status in A: *ICAM* and B: *RASSF1a* promoters. In A2780 (A, n=3) and MCF7 (B, n=1) cells expressing the specified constructs, *ICAM* and *RASSF1*a, CpG sites methylation status were quantified. CpG sites in *ICAM* promoter were selected based on Chen et al. report. NED: no effector domain. Two-tailed unpaired t-test; error bars±s.d

Targeted HBx induces re-expression of silent *PLOD2*

HBx is known for altering expression of several cellular genes by indirect mechanisms. To address a direct transcriptional upregulatory function of HBx, we evaluated the impact of HBx on a repressed and hypomethylated gene taking advantage of the CRISPR/dCas9 targeting ability. To do so, we used a previously reported methodology using four single guide RNAs (sgRNAs) targeting the *PLOD2* gene promoter in C33A cells (S4 Table) [31], along with the dCas9 protein alone (NED) or fused to either VP64 or HBx.

Figure 3 shows that VP64-induced *PLOD2* mRNA expression ranged from 3 times with sgRNA 2 to 300 times with a combination of all sgRNAs, in concordance with previous reports where multiple sgRNAs are more effective than individual sgRNAs. HBx induction using the independent sgRNAs did not increase *PLOD2* mRNA expression. Interestingly, a combination of all sgRNAs successfully increased *PLOD2* gene expression compared to the irrelevant sgRNA control (1.9 times; $p<0.05$).

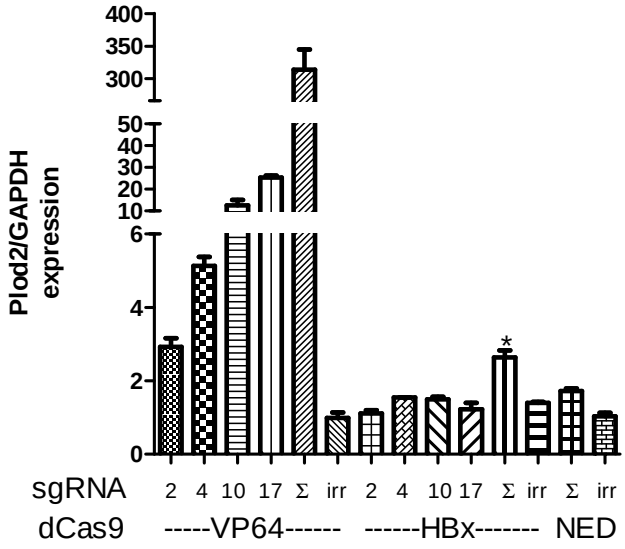

Figure 3. dCas9-HBx activates *PLOD2* expression. pdCas9-VP64 or -HBx fused vectors were transfected along with sgRNAs 2, 4, 10, 17 independently or a combination of all sgRNAs targeting *PLOD2* gene promoter. *PLOD2* mRNA expression was evaluated 48 h pt and normalized against *GAPDH* expression. NED: No effector domain. Irr: no *PLOD2* directed sgRNA. Two-tailed unpaired t-test, *P<0.05, n=3 independent experiments; error bars±s.d

CHAPTER 6

Discussion

In this study, we evaluated the effect of HBx on host DNA methylation signatures and targeted expression modulation. We could observe induction of expression of a DNA methylation-independent repressed gene but were unsuccessful in demonstrating HBx as a potential epigenetic modulator useful for EGE.

The HBx protein is a known multitarget protein, able to alter a number of cellular functions as signal transduction, cell cycle regulation and epigenetic control of both cellular and viral genome (35). Although the exact mechanism of action of HBx is unclear, HBx is considered an important player in causing carcinoma and alteration in the host DNA methylation status has been widely recognized in hepatocellular carcinoma. Indeed, in this cancer type altered DNMT expression and function are known to result in both genome-wide hypomethylation and site-specific DNA hypermethylation simultaneously (36).

Accordingly, Park el at., reported a significantly lower genome-wide methylation level in HBx-positive HCC cases; compared with HBx-negative HCC cases (21). In contrast, Okamoto reported increased DNA methylation for 338/407 genes in HBV-relates HCC cases expressing HBx (37). For human hepatocyte chimeric mice, a time-dependent methylation increase was observed for five selected genes (*RASSF1a*, Estrogen receptor 1 (*ESR1*), homeobox A6 (*HOXA6*), zinc-finger protein 385A and ELOVL fatty acid elongase 3),

and for more than 160 genes (in a 542 array) as measured by methylated CpG island amplification-microarray (MCAM) (37).

Although the mechanism for the HBx-related altered DNA methylation status is still largely unknown, it has been shown that HBx can induce DNMT3B downregulation, and stimulation of expression of DNMT1, DNMT3a1, DNMT3a2 [23,41,42]. Additionally, HBx interacts with DNMT3a1 and DNMT3a2 disturbing their activities, inducing its redistribution to other promoters; leading to aberrant hyper- and hypomethylation in specific regions of the cellular genome (21, 26, 38, 39). To further address this issue, we evaluated the global methylation status of HepG2 cells in the presence and absence of HBx protein.

In our hands, at the evaluated time points, we could not detect altered methylation of the cellular genome. The absence of a short-term effect of HBx expression can be explained by the fact that HBV-related liver disease as cirrhosis and HCC take decades to be developed (36, 39-41). As it is possible that the evaluated time points are not enough for successful whole genome epigenetic alterations, we can not conclude that HBx does not affect DNA methylation.

The experimental approach used in this study can be used in further experiments to ensure the controlled expression of HBx for longer periods, which could be used to evaluate the HBx effect not only in DNA methylation but also histone PTM alterations. In this respect, HBx has been shown to prevent and reverse the viral chromatin

deacetylation by inhibiting HDAC1 (17, 26). As a consequence, deposition of repressive marks H3K9me3 and H3K9me2, which recruit silencing proteins like HP1 and SETDB1 is attenuated (19, 20). Additionally, recruitment of LSD1 (related with reduction of H3K9me) and Set1A (promotes H3K4me3) to the viral promoters leads to the increase of in histone acetylation and H3K4me deposition (42).

Considering the documented effect of HBx on the upregulation of viral and cellular gene expression (17, 20, 43, 44), we intended to evaluate the role of HBx by fusing HBx with two previously reported zinc-finger proteins (CD54 and ZF2, targeting the *ICAM1* or *RASSF1a* promoter regions, respectively (31, 33).

The evaluated ZF protein CD54, directed against the CpG island of the *ICAM1* gene promoter, downstream of the TSS, binds the 10^{th} and 11^{th} position of the interrogated CpG region (34). Meanwhile ZF2 targets the CpG site 10^{th} of the island associated with the *RassF1a* gene promoter (counting 32 CpG sites long the island) upstream the TSS of the *RassF1* mRNA (31, 45)

In contrast to a previous study, in which ICAM1 promoter demethylation was reported using ZF CD54 fused to TET2 (as also for another EpCAM-targeting ZF) (34), we could not obtain significant demethylation for the previously reported CpG sites of ICAM1 nor for the analyzed region of the RASSF1a promoter utilizing the 5mC oxidation inducer TET2, the transcription activator VP64 or HBx. In our previous study, we observed efficient lowering of methylation at the CpG sites #10 and #11 which were directly bound by the ZF

CD54, even in the absence of an effector domain. Taking into account that also ZF2 directly targets a CpG site, and that for both constructs we did not observe an effect on these directly targeted CpGs, we conclude that the experimental set up was sub-optimal and that, for example, the sorting of GFP positive cells should be more stringent.

Although HBx protein lacks sequence specific targeting ability, HBx has been associated with recruitment of transcription factors, activator proteins and transcription machinery leading to upregulation of cellular and viral genes during HBV infection (17, 46-48). In this study, we thus evaluated the ability of HBx to induce targeted transcription re-expression on a methylation-independent epigenetically silenced gene. For this purpose we choose the procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) gene, which is transcriptionally repressed in C33A, while its promoter has very low DNA methylation levels (31).

We took advantage of the CRISPR/dCas9 system and previously reported four sgRNAs (31). Independent sgRNAs successfully increased *PLOD2* mRNA expression using VP64 and this upregulation was further enhanced by using a combination of several sgRNAs, as also reported by others (49-51). Contrary to VP64, HBx failed to substantially induce *PLOD2* expression using independent sgRNAs. However, it successfully induced a slight but significant increase in gene expression utilizing a combination of all sgRNAs (**Fig 3**).

At this point, chromatin immunoprecipitation assay (ChIP) could be used to identify other proteins being recruited by HBx which might explain the activation of *PLOD2* promoter.

Based on our results, targeting of HBx to the *PLOD2* promoter exerted an upregulatory effect in presence of a mix of sgRNAs which successfully overcame the repressive mechanisms acting on this promoter in C33A cells.

During HBV infection, HBx is recruited to the viral or cellular chromatin by interaction with cellular proteins as transcription factors and cofactors, from where HBx can recruit other proteins (48). Additionally, functional studies involving wild type HBx and mutant variants could directly relate wt HBx and the presence of epi-proteins associated with active chromatin. Interestingly, truncated HBx and mutants as Lys-130 and Val-131, which could be used as negative controls for HBx activity, indeed fail to recruit those "activating" epi-proteins and the presence of epi-proteins related to inactive chromatin is enhanced (17, 26, 52). In this regard, we speculate that the presence of HBx can activate transcription by recruiting transcription factors while preventing epigenetic inactivation.

Epigenetic editing in general intends to modify epigenetic marks on DNA and histones in order to control the expression of any desired gene; in this sense, epigenetic editing tools consist of a DNA binding domain (DBD) and an epigenetic effector defined as a writers or erasers with catalytic activity. Among the recognized DBD used to target specific DNA sequences are the zinc-finger proteins (ZFPs), triplex forming helix (TFO), transcription activator like domains (TALEs) and clustered regularly interspaced short palindromic repeats (CRISPRs) (53-55).

Various epigenetic effector domains, including DNA methyltransferases (human and mouse DNMTs, M.HhaI and M.HpaII), DNA demethylases (TDG and TET proteins) and histone posttranslational modifiers have been previously reported and used in EGE experiments (53). In addition to these catalytically active epigenetic modifiers, other kinds of proteins with transcriptional activator or repressor activity can be used as part of the tool set to modulate gene expression, including Krab/SKD and VP16/VP64.

HBx might be an interesting addition as epigenetic editing tool due to its known epigenetic effects on the cellular and viral genome (36) and its small size. Indeed, effective cloning and expression of large sequences of effector domains into experimental delivery systems (56), as well as accessibility in heterochromatin, is oftentimes presenting a major challenge for the commonly used Cas9 and TALE models (57).

Taken together, although in this study at the evaluated times, HBx failed to induce targeted site specific demethylation or global methylation changes, HBx was able to induce targeted gene re-expression. Due to the limited time periods evaluated by us, we cannot rule out possible aberrant DNA methylation inductions related to HBx. As effects of HBx through DNA methylation changes could be an accumulative process, longer HBx exposure periods might alter the DNA methylation homeostasis by affecting epi-enzyme expression or by direct interaction with those proteins.

Our results suggest that HBx locally induces a transcriptionally permissive status of the chromatin, probably by recruiting proteins related to active epigenetic marks and

transcription machinery. Although this result needs further validation using other cellular and gene models, the usage of this protein is promising in models where a weak induction could be preferred against the generally strong over-induction observed with the commonly used VP64.

In conclusion, HBx should be further considered as a potential targeted inducer of gene expression for (non-methylated) repressed genes, and possible epigenetic alterations should not yet be discarded.

References

1. www.who.int. World Health Organization, WHO. Hepatitis B. [Internet] Geneva: WHO; 2015 [cited 2015 Oct-10-2015]; Available from: http://www.who.int/mediacentre/factsheets/fs204/en/.
2. Dufour JF, Johnson P. Liver cancer: from molecular pathogenesis to new therapies: summary of the EASL single topic conference. J Hepatol. 2010;52(2):296-304.
3. Beasley RP, Hwang LY, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22 707 men in Taiwan. Lancet. 1981;2(8256):1129-33.
4. Feitelson MA, Lee J. Hepatitis B virus integration, fragile sites, and hepatocarcinogenesis. Cancer letters. 2007;252(2):157-70.
5. Lupberger J, Hildt E. Hepatitis B virus-induced oncogenesis. World J Gastroenterol. 2007;13(1):74-81.
6. Wang HC, Huang W, Lai MD, Su IJ. Hepatitis B virus pre-S mutants, endoplasmic reticulum stress and hepatocarcinogenesis. Cancer Sci. 2006;97(8):683-8.
7. Lee YI, Hwang JM, Im JH, Kim NS, Kim DG, Yu DY, et al. Human hepatitis B virus-X protein alters mitochondrial function and physiology in human liver cells. J Biol Chem. 2004;279(15):15460-71.
8. Benhenda S, Cougot D, Buendia MA, Neuveut C. Hepatitis B virus X protein molecular functions and its role in virus life cycle and pathogenesis. Adv Cancer Res. 2009;103:75-109.
9. Keasler VV, Hodgson AJ, Madden CR, Slagle BL. Enhancement of hepatitis B virus replication by the regulatory X protein in vitro and in vivo. J Virol. 2007;81(6):2656-62.
10. Feitelson MA, Reis HM, Liu J, Lian Z, Pan J. Hepatitis B virus X antigen (HBxAg) and cell cycle control in chronic infection and hepatocarcinogenesis. Front Biosci. 2005;10:1558-72.
11. Arbuthnot P, Capovilla A, Kew M. Putative role of hepatitis B virus X protein in hepatocarcinogenesis: effects on apoptosis, DNA repair, mitogen-activated protein kinase and JAK/STAT pathways. J Gastroenterol Hepatol. 2000;15(4):357-68.
12. Liu XH, Lin J, Zhang SH, Zhang SM, Feitelson MA, Gao HJ, et al. COOH-terminal deletion of HBx gene is a frequent event in HBV-associated hepatocellular carcinoma. World J Gastroenterol. 2008;14(9):1346-52.
13. Garcia-Saez I, Lacroix FB, Blot D, Gabel F, Skoufias DA. Structural characterization of HBXIP: the protein that interacts with the anti-apoptotic protein survivin and the oncogenic viral protein HBx. J Mol Biol. 2011;405(2):331-40.
14. Rawat S, Bouchard MJ. The hepatitis B virus (HBV) HBx protein activates AKT to simultaneously regulate HBV replication and hepatocyte survival. J Virol. 2015;89(2):999-1012.
15. Ma J, Sun T, Park S, Shen G, Liu J. The role of hepatitis B virus X protein is related to its differential intracellular localization. Acta Biochim Biophys Sin (Shanghai). 2011;43(8):583-8.
16. Xie N, Chen X, Zhang T, Liu B, Huang C. Using proteomics to identify the HBx interactome in hepatitis B virus: how can this inform the clinic? Expert Rev Proteomics. 2014;11(1):59-74.
17. Belloni L, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M, et al. Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. Proc Natl Acad Sci U S A. 2009;106(47):19975-9.

18. Luo L, Chen S, Gong Q, Luo N, Lei Y, Guo J, et al. Hepatitis B virus X protein modulates remodelling of minichromosomes related to hepatitis B virus replication in HepG2 cells. Int J Mol Med. 2013;31(1):197-204.
19. Protzer U. Hepatitis: Epigenetic control of HBV by HBx protein--releasing the break? Nat Rev Gastroenterol Hepatol. 2015;12(10):558-9.
20. Riviere L, Gerossier L, Ducroux A, Dion S, Deng Q, Michel ML, et al. HBx relieves chromatin-mediated transcriptional repression of hepatitis B viral cccDNA involving SETDB1 histone methyltransferase. J Hepatol. 2015;63(5):1093-102.
21. Park IY, Sohn BH, Yu E, Suh DJ, Chung YH, Lee JH, et al. Aberrant epigenetic modifications in hepatocarcinogenesis induced by hepatitis B virus X protein. Gastroenterology. 2007;132(4):1476-94.
22. Benn J, Schneider RJ. Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. Proc Natl Acad Sci U S A. 1994;91(22):10350-4.
23. Dewantoro O, Gani RA, Akbar N. Hepatocarcinogenesis in viral Hepatitis B infection: the role of HBx and p53. Acta Med Indones. 2006;38(3):154-9.
24. Rouleau J, MacLeod AR, Szyf M. Regulation of the DNA methyltransferase by the Ras-AP-1 signaling pathway. J Biol Chem. 1995;270(4):1595-601.
25. Truant R, Antunovic J, Greenblatt J, Prives C, Cromlish JA. Direct interaction of the hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response element-directed transactivation. J Virol. 1995;69(3):1851-9.
26. Zheng DL, Zhang L, Cheng N, Xu X, Deng Q, Teng XM, et al. Epigenetic modification induced by hepatitis B virus X protein via interaction with de novo DNA methyltransferase DNMT3A. J Hepatol. 2009;50(2):377-87.
27. Lucifora J, Arzberger S, Durantel D, Belloni L, Strubin M, Levrero M, et al. Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. J Hepatol. 2011;55(5):996-1003.
28. Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, et al. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. Gastroenterology. 2006;130(3):823-37.
29. Falahi F, van Kruchten M, Martinet N, Hospers GA, Rots MG. Current and upcoming approaches to exploit the reversibility of epigenetic mutations in breast cancer. Breast Cancer Res. 2014;16(4):412.
30. Jurkowski TP, Ravichandran M, Stepper P. Synthetic epigenetics-towards intelligent control of epigenetic states and cell identity. Clin Epigenetics. 2015;7:18.
31. Cano-Rodriguez D, Gjaltema RA, Jilderda LJ, Jellema P, Dokter-Fokkens J, Ruiters MH, et al. Writing of H3K4Me3 overcomes epigenetic silencing in a sustained but context-dependent manner. Nat Commun. 2016;7:12284.
32. Falahi F, Huisman C, Kazemier HG, van der Vlies P, Kok K, Hospers GA, et al. Towards sustained silencing of HER2/neu in cancer by epigenetic editing. Mol Cancer Res. 2013;11(9):1029-39.
33. Huisman C, van der Wijst MG, Falahi F, Overkamp J, Karsten G, Terpstra MM, et al. Prolonged re-expression of the hypermethylated gene EPB41L3 using artificial transcription factors and epigenetic drugs. Epigenetics. 2015;10(5):384-96.

34. Chen H, Kazemier HG, de Groote ML, Ruiters MH, Xu GL, Rots MG. Induced DNA demethylation by targeting Ten-Eleven Translocation 2 to the human ICAM-1 promoter. Nucleic Acids Res. 2014;42(3):1563-74.
35. Kumar V, Sarkar DP. Hepatitis B Virus X Protein: Structure–Function Relationships and Role in Viral Pathogenesis. In: Goosen M, Kaufmann J, Triezenberg SJ, editors. Transcription Factors: Springer-Verlag; 2004. p. 377-407.
36. Tian Y, Yang W, Song J, Wu Y, Ni B. Hepatitis B virus X protein-induced aberrant epigenetic modifications contributing to human hepatocellular carcinoma pathogenesis. Mol Cell Biol. 2013;33(15):2810-6.
37. Okamoto Y, Shinjo K, Shimizu Y, Sano T, Yamao K, Gao W, et al. Hepatitis virus infection affects DNA methylation in mice with humanized livers. Gastroenterology. 2014;146(2):562-72.
38. Lee SM, Lee YG, Bae JB, Choi JK, Tayama C, Hata K, et al. HBx induces hypomethylation of distal intragenic CpG islands required for active expression of developmental regulators. Proc Natl Acad Sci U S A. 2014;111(26):9555-60.
39. Zhao J, Wu G, Bu F, Lu B, Liang A, Cao L, et al. Epigenetic silence of ankyrin-repeat-containing, SH3-domain-containing, and proline-rich-region- containing protein 1 (ASPP1) and ASPP2 genes promotes tumor growth in hepatitis B virus-positive hepatocellular carcinoma. Hepatology. 2010;51(1):142-53.
40. Yang HI, Yeh SH, Chen PJ, Iloeje UH, Jen CL, Su J, et al. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. J Natl Cancer Inst. 2008;100(16):1134-43.
41. Koike K. Hepatitis B virus X gene is implicated in liver carcinogenesis. Cancer Lett. 2009;286(1):60-8.
42. Alarcon V, Hernandez S, Rubio L, Alvarez F, Flores Y, Varas-Godoy M, et al. The enzymes LSD1 and Set1A cooperate with the viral protein HBx to establish an active hepatitis B viral chromatin state. Sci Rep. 2016;6:25901.
43. van Breugel PC, Robert EI, Mueller H, Decorsiere A, Zoulim F, Hantz O, et al. Hepatitis B virus X protein stimulates gene expression selectively from extrachromosomal DNA templates. Hepatology. 2012;56(6):2116-24.
44. Wu CG, Salvay DM, Forgues M, Valerie K, Farnsworth J, Markin RS, et al. Distinctive gene expression profiles associated with Hepatitis B virus x protein. Oncogene. 2001;20(28):3674-82.
45. Loginov VI, Maliukova AV, Seregin Iu A, Khodyrev DS, Kazubskaia TP, Ermilova VD, et al. [Methylation of the promoter region of the RASSF1A gene, a candidate tumor suppressor, in primary epithelial tumors]. Mol Biol (Mosk). 2004;38(4):654-67.
46. Barnabas S, Hai T, Andrisani OM. The hepatitis B virus X protein enhances the DNA binding potential and transcription efficacy of bZip transcription factors. J Biol Chem. 1997;272(33):20684-90.
47. Cha MY, Ryu DK, Jung HS, Chang HE, Ryu WS. Stimulation of hepatitis B virus genome replication by HBx is linked to both nuclear and cytoplasmic HBx expression. J Gen Virol. 2009;90(Pt 4):978-86.
48. Cougot D, Wu Y, Cairo S, Caramel J, Renard CA, Levy L, et al. The hepatitis B virus X protein functionally interacts with CREB-binding protein/p300 in the regulation of CREB-mediated transcription. J Biol Chem. 2007;282(7):4277-87.

49. Maeder ML, Linder SJ, Cascio VM, Fu Y, Ho QH, Joung JK. CRISPR RNA-guided activation of endogenous human genes. Nature methods. 2013;10(10):977-9.
50. Gilbert LA, Larson MH, Morsut L, Liu Z, Brar GA, Torres SE, et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell. 2013;154(2):442-51.
51. Konermann S, Brigham MD, Trevino AE, Joung J, Abudayyeh OO, Barcena C, et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature. 2015;517(7536):583-8.
52. Gong Q, Chen S, Guo J, Sun H, Zheng G, Liu Q, et al. Chromosome remodeling related to hepatitis B virus replication in HepG2 cells. DNA Cell Biol. 2011;30(6):347-54.
53. de Groote ML, Verschure PJ, Rots MG. Epigenetic Editing: targeted rewriting of epigenetic marks to modulate expression of selected target genes. Nucleic Acids Res. 2012;40(21):10596-613.
54. Ding Y, Li H, Chen LL, Xie K. Recent Advances in Genome Editing Using CRISPR/Cas9. Front Plant Sci. 2016;7:703.
55. Gaj T, Gersbach CA, Barbas CF, 3rd. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. Trends Biotechnol. 2013;31(7):397-405.
56. Jurkowski TP, Ravichandran M, Stepper P. Synthetic epigenetics-towards intelligent control of epigenetic states and cell identity. Clin Epigenetics. 2015;7(1):18.
57. Kungulovski G, Jeltsch A. Epigenome Editing: State of the Art, Concepts, and Perspectives. Trends Genet. 2016;32(2):101-13.

Supplemantary Information

Table S1. List of primers used for PCR and qRT-PCR

Name	Sequence 5´->3
HBx-pMX-Fw	GAGAACGCGTATGGCTGCTCGGTTGTGCTGCCAA
HBx.2-Rv	GAGACTCGAGTTCTTAATTAATTATCGTCGTCATCCTTGTAATCT CCTGATCCGGCAGAGGTGGTGAAAAAGAAGCATGCTGCAGGT GAACAGACCAA
PLOD2-Fw	GGGAGTTCATTGCACCAGTT
PLOD2-Rv	GAGGACGAAGAGAACGC
GAPDH-Fw	CCACATCGCTCAGACACCAT
GAPDH-Rv	GCGCCCAATACGACCAAAT
HBx-qPCR-Fw	CGTCTGTGCCTTCTCATCTG
HBx-qPCR-Rv	GTCCTCTTGTGTAAGACCTTGG

Table S2. Zinc finger recognition sites

ZINC-FINGER	RECOGNITION SITE 5´->3´
ICAM (CD54)	TCCGGAGCTGAAGCGGCC
RASSF1 (ZF2)	GGAGGGGACGAAGGAGGG

S3 Table. Pyrosequencing primers

Target	Forward primer	Reverse primer	Pyrosequencing
ICAM1	GGGGAAGTTGGTAGTATTTAAAAGT	[Btn] CCTTCCCCTCCCAAACAAATACTACAATTA	GTTAGATTGTTTTAGT
RASSf1a	AAGGAGGGAAGGAAGGGTAAG	GGGACACCGCTGATCGTTTACCCCCAACTCAATAAACTCAAACTCCCC	GAAGGAAGGGTAAGG
Universal	[Btn] GGGACACCGCTGATCGTTTA		

S4 Table. *PLOD2* targeting sgRNAs sequences

SgRNA	Sequence 5´->3´
PLOD2 g1	CCACTCCCAAAGCTAAGTGC
PLOD2 g2	GCTGTGGAAGCTACCGGGGC
PLOD2 g4	GAGCCTCCACACGTAGCCGC
PLOD2 g5	TGAGCAAACAGTCCAGACGT